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14. ABSTRACT
During the first year of this innovator award, we made significant progress toward two of our aims. We constructed a third generation RNAi library and made that available to the breast cancer community. This resource has nearly 75,000 independent, sequence verified clones targeting ~18,000 human genes. A similar library for the mouse genome is nearing completion. We also scaled up our shRNA screening platform in preparation for lethality surveys of all suitable and available BC cell lines, including matched pairs of lines that have acquired resistance to herceptin in vitro. Relevant to our second aim, we have profiled microRNA from each of the identifiable epithelial cell types in the mouse mammary gland and are undertaking similar efforts in human. The goal is to develop microRNA sensor strategies that will permit visualization of each cell type in vivo and enable their isolation and manipulation in vitro. Finally, we showed that two microRNAs, let-7 and miR-93, can deplete tumor initiating cells from a number of basal breast cancer cell lines.

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Introduction

The goal of this innovator award is to continue to develop and apply RNAi-based screening methods to discover new routes toward breast cancer therapy. The project has three sets of goals. First is to integrate genomic and genetic information on available breast cancer cell lines to identify tumor-specific vulnerabilities and to understand genetic determinants of therapy resistance. Second is to probe the roles of breast cancer stem cells, with a particular emphasis on microRNAs. The third is to examine regions that determine familial susceptibility to breast cancer by applying novel, focal re-sequencing methods developed in the laboratory.

Body

The first year of this innovator award has set the stage for future studies and has seen progress on all areas of scientific focus. Relevant to the first aim, our studies have been focused in three general areas. The first was based upon the realization that we had learned a tremendous amount about the RNAi pathway in the several years since we had constructed our shRNA library version 2. We decided to take advantage of those developments to construct improved tools as a prelude to large-scale genetic studies. This required the development of new technologies for sequencing as well as a massive effort at library construction. Ultimately, this resulted in the generation of approximately 75,000 new shRNA clones corresponding to ~18,000 human genes. These have now been sequence-verified and are available to the entire breast cancer research community through Open Biosystems. A second major goal was to collect the substrate for our screening efforts. This entailed bringing to CSHL approximately 100 cell lines that represent a spectrum of breast cancers, including ER-positive disease, HER-2 amplified lines, and triple-negative disease. We have collected IC20 and IC80 data on sensitivity to the appropriate agents (lapatinib, herceptin, tamoxifen, and fulvestrant). We have also devoted major efforts to industrializing the shRNA screening process. Both library virus production and cell screening can now be carried out in hyperflasks, enabling full genome screens of four cell lines per person simultaneously. Within four weeks, we will initiate the full scale screening program to survey vulnerabilities and modifiers of drug sensitivity in as many cell lines within the full panel as grow at sufficient rates to enable screening. We have also collected from Dennis Slamon two pairs of cell lines that were initially sensitive to trastuzumab but that have been conditioned to resistance. These have been screened at a small scale (reduced representation set of 10,000 clones of the V2 library) and that data is currently being analyzed. They will be screened genome-wide with the V3 collection.

We recently demonstrated that one microRNA, miR-451, has an unusual biogenesis mechanism that does not require Dicer and that leaves no passenger strand. We are exploring the possibility that miR-451 will serve as an improved microRNA scaffold for more effective RNAi and will incorporate this into the BCRP funded program if warranted in the next year.

Relevant to Aim 2, we have continued our characterization of mammary stem cells and breast cancer initiating cells from several standpoints. First, we have worked with Max Wicha to show that even transient expression of miR-93 or let-7 can deplete tumor initiating cells from several basal human breast cancer cell lines. Perhaps the most striking experiments are those in which microRNA expression is activated during tumor growth. In these studies, the primary tumor continues to expand, though its growth is somewhat slowed. However, the tumors can no longer be transplanted to secondary recipients. Thus, there is a "memory" of exposure to these microRNAs that is most likely manifested as a loss of tumor initiating cells. Strikingly, expression of let-7 or miR-93 can completely prevent tumor formation if cells are exposed prior to orthotopic transplantation or tail vein injection to seed metastatic sites. Given this success, we have begun to build sensors for let-7 and mir-93 that will not simply mark cells that lack expression of these miRNAs but also sensors that provide functionality. Specifically, we have constructed Cre and diptheria toxin receptor (DTR) transgenes with microRNA binding sites in their 3' UTRs. These have just been transferred into commaD cells to test their selective function in the stem cell compartment. If these prove efficient, we can then pursue in vivo studies where oncogenic manipulations can be carried out selectively in stem cell compartments and where stem cell compartments can be selectively ablated during tumor progression, metastasis, or in combination with conventional therapies. We have also followed a second course, taking advantage of the let-7 sensor to perform a reduced representation RNAi screen for shRNAs that deplete the stem cell compartment. This has served mainly as a proof of principle to full genome studies with the V3 library. A number of positive hits have been identified from the primary screen, including components in known self renewal pathways (e.g. Notch pathway). These are being confirmed at present by testing individually in colony formation and repopulation experiments. A third goal has been to better characterize the stem cell compartment to derive better markers for following repopulating cells and potentially also cancer stem cells. Based upon the best available markers, we have isolated 7 different populations of mammary epithelial cells and have profiled their mRNA and miRNA populations. We performed similar studies in histone H2B GFP "label retaining" cells from the mammary gland. Focusing first on the miRNAs, we have designed Boolean microRNA expressions that describe each cell fate. We are presently verifying expression profiles initially determined by sequencing using qPCR methods. In parallel, we are constructing sensors that we hope will allow us to precisely monitor, isolate, and manipulate each epithelial cell type in the gland.
Progress relevant to Aim 3 has been more modest in the first year and has mainly been restricted to the development of new analysis pipelines for calling variation in captured genomic sequence. However mundane, this was absolutely necessary because no real standards have yet been established for making variation calls in next generation sequencing.

Key Research Accomplishments

1. sequence validation and release of a third-generation shRNA library
2. collection of cell lines for screening within the proposed program

3. determination that microRNAs can impact the tumor initiating compartment in human breast cancer cell lines
4. expression profiling of mammary stems cells and the identification or candidate marker strategies for their more precise isolation
5. construction of data analysis pipelines for variant calling in next-generation sequencing data

Reportable outcomes

Rosa-Rosa JM, Gracia-Aznárez FJ, Hodges E, Pita G, Rooks M, Xuan Z, Bhattacharjee A, Brizuela L, Silva JM, Hannon GJ, Benitez J. Deep sequencing of target linkage assay-identified regions in familial breast cancer: methods, analysis pipeline and troubleshooting. (2010) PLoS One. 5(4):e9976.
V3 shRNA library corresponding to 75,000 plasmids

Conclusions

Overall, we have made significant progress on two of the proposed aims in the first year of this award and have set the stage for identifying new therapeutic targets for breast cancer and for specifically targeting the tumor initiating compartment in mammary tumors.